Reference 1

were unable to use this method for one of 41 sites, since the responses were too small. For this site, the onset of eye movements was detected visually.

For each condition and each stimulation site, the gain of the responses to the perturbations was computed as the square root of E divided by T, where E is the area within the polygon defined by the polar plot for each set of eight perturbations in different directions, and T is the area within the polygon defined by the peak target velocity, always 282.84.

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A role for ghrelin in the central regulation of feeding

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Ghrelin is an acylated peptide that stimulates the release of growth hormone from the pituitary[1]. Ghrelin-producing neurons are located in the hypothalamus, whereas ghrelin receptors are expressed in various regions of the brain[2–4], which is indicative of central—and as yet undefined—physiological functions. Here we show that ghrelin is involved in the hypothalamic regulation of energy homeostasis. Intracerebroventricular injections of ghrelin strongly stimulated feeding in rats and increased body weight gain. Ghrelin also increased feeding in rats that are genetically deficient in growth hormone. Anti-ghrelin immunoglobulin G robustly suppressed feeding. After intracerebroventricular ghrelin administration, Fos protein, a marker of neuronal activation[5], was found in regions of primary importance in the regulation of feeding, including neuropeptide Y[6] (NPY) neurons and agouti-related protein[7] (AGRP) neurons. Antibodies and antagonists of NPY and AGRP abolished ghrelin-induced feeding. Ghrelin augmented NPY gene expression and blocked leptin-induced[8] feeding

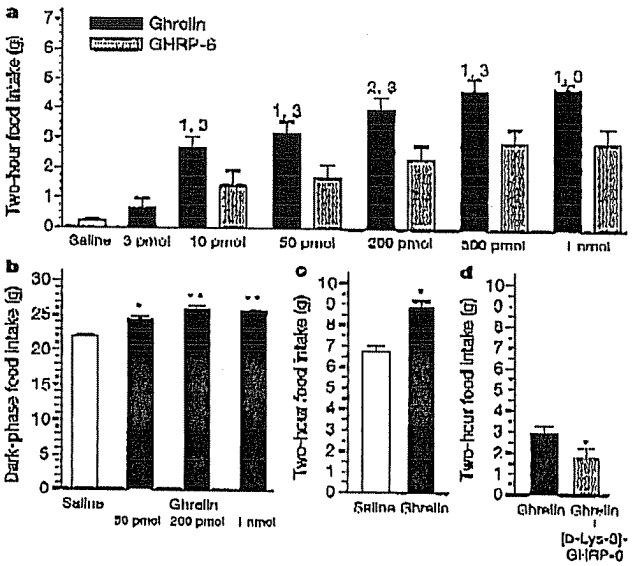

Figure 1 Stimulation of feeding by single ICV administration of ghrelin. **a**, Two-hour food intake (mean ± s.e.m.) of free-feeding rats injected with various doses of ghrelin or GHRP-6. Control rats were given 0.9% saline. ANOVA was only performed on the ghrelin group against the control group. 1, $P < 0.05$ versus GHRP-6; 2, $P < 0.01$ versus GHRP-6; 3, $P < 0.0001$ versus saline. **b**, Dark-phase (19:00–07:00) food intake of rats receiving an ICV administration of ghrelin at 18:45. Asterisk, $P < 0.005$; double asterisk, $P < 0.001$. **c**, 2-h food intake of 8-h fasted rats receiving ghrelin (200 pmol) at 08:45. Asterisk, $P = 0.0001$. **d**, Suppressive effect of [D-Lys-3]-GHRP-6 (5 nmol) on feeding induced by ghrelin (20 pmol). Asterisk, $P = 0.012$.

reduction, implying that there is a competitive interaction between ghrelin and leptin in feeding regulation. We conclude that ghrelin is a physiological mediator of feeding, and probably has a function in growth regulation by stimulating feeding and release of growth hormone.

Ghrelin increased the food intake of rats in both satiated and feeding conditions. Intracerebroventricular (ICV) administration of ghrelin above a minimally active dose of 10 pmol to free-feeding rats during the early light phase (satiated) increased food intake in a dose-dependent manner (Fig. 1a). Ghrelin-treated rats showed no unusual behaviour relative to the controls. Administration of ghrelin also significantly increased dark phase (feeding) food intake (Fig. 1b). In rats that had fasted for 8 h, ghrelin also increased their 2-h food intake relative to the saline-injected group (Fig. 1c).

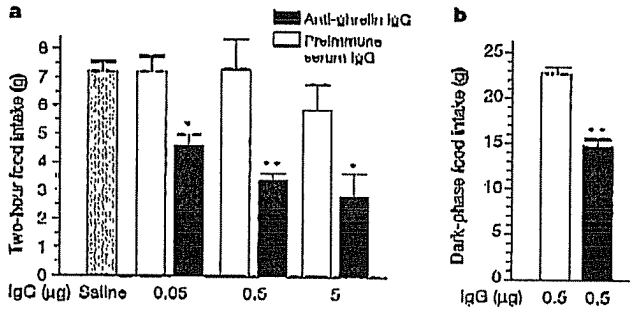

Figure 2 Anti-ghrelin IgG suppresses feeding. **a**, Food intake of rats that had fasted for 8 h, then received ICV administration of 0.05, 0.5 or 5 μg anti-ghrelin IgG or preimmune serum IgG at 08:45. **b**, Dark-phase food intake of free-feeding rats that received ICV administration of 0.5 μg anti-ghrelin IgG or preimmune serum IgG at 17:00. Asterisk, $P < 0.005$; double asterisk, $P < 0.0001$ versus preimmune IgG.

Centrally administered ghrelin induced feeding behaviour within 5 min of administration. GHRP-6 (modelled after enkephalins) is a synthetic hexapeptide that binds to growth hormone secretagogue receptor (GHS-R), releases growth hormone[9] and stimulates feeding[10]. ICV-injected ghrelin was more effective at stimulating food intake than GHRP-6 (Fig. 1a). Ghrelin-induced feeding was suppressed by an antagonist for GHS-R[11], [D-Lys-3]-GHRP-6 (Fig. 1d).

To determine whether an endogenous tone of ghrelin signalling is present in the hypothalamus, we investigated the effect of an antibody against ghrelin on feeding behaviour. Compared with the preimmune serum immunoglobulin G (IgG), anti-ghrelin IgG suppressed starvation-induced feeding in a marked, dose-dependent manner (Fig. 2a). Anti-ghrelin IgG also suppressed dark phase food intake by 36% in free-feeding rats (Fig. 2b). These findings indicate that ghrelin is a powerful, endogenous orexigenic peptide.

A chronic ICV infusion of ghrelin (250 pmol d^{-1}) for 12 d using an osmotic minipump increased food intake and body weight gain over the infusion period (Fig. 3). It did not affect general activity (ghrelin: dark phase, 96 ± 6% of control activity; light phase, 95 ± 8%; $P = 0.5$), indicating that ghrelin does not mediate nonspecific arousal. The plasma concentrations of glucose, insulin, triglycerides and total cholesterol in the ghrelin-infused group did not differ from those in the control group (data not shown).

ICV-injected ghrelin also stimulated food intake in spontaneous dwarf rats (SDR)[12], a growth-hormone-deficient rat model that carries a disrupted growth-hormone gene[13] (food intake: 200 pmol ghrelin, 1.18 ± 0.10 g; vehicle, 0.01 ± 0.01 g; $P < 0.0001$). Thus, the stimulatory effect of ghrelin on feeding does not depend on the stimulation of growth hormone.

To establish the neuronal populations activated by central ghrelin, we mapped c-*fos* expression after an ICV administration of ghrelin. Fos-immunoreactive neurons were observed primarily in regions implicated in the regulation of feeding behaviour (Fig. 4). This distribution is coincident with that of GHS-R[4], which is also

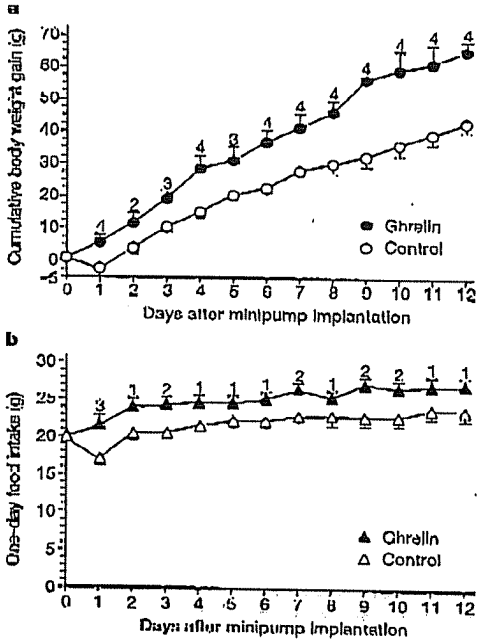

Figure 3 Effect of chronic ghrelin ICV administration on rats. Cumulative body weight gain (a) and one-day food intake (b) during an ICV infusion of 250 pmol d^{-1} for 12 d. Alzet minipumps were implanted on day 0. 1, $P < 0.05$; 2, $P < 0.01$; 3, $P < 0.005$; 4, $P < 0.001$.

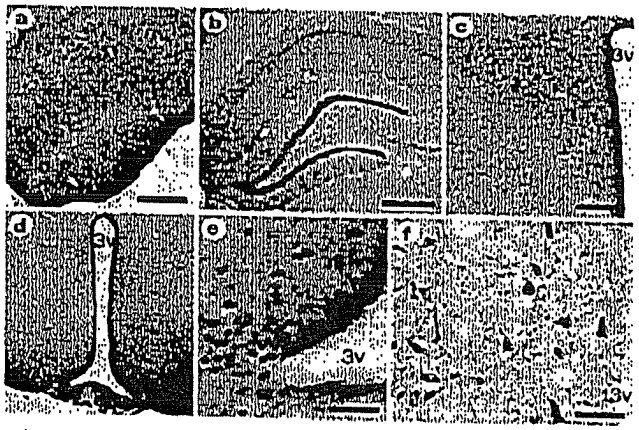

Figure 4 Localization of Fos expression in response to ICV administration of ghrelin. **a**, Piriform cortex. **b**, Dentate gyrus and hippocampus. **c**, Paraventricular nucleus. **d**, Arcuate, dorsomedial and ventromedial hypothalamic nuclei. **e**, High magnification of Fos immunoreactivity in the arcuate nucleus (magn. × 200). **f**, Co-staining of Fos (blue-black) and NPY neurons (brown) in the arcuate nucleus. All sections are from a rat given 0.5 nmol ghrelin. Fos is also found in the olfactory nerve layer; granular cell layer of the olfactory bulb; insular, prelimbic, infralimbic, orbital and cingulate cortices; accumbens, lateral septal, paraventricular thalamic, periventricular hypothalamic, anterior hypothalamic, supraoptic, suprachiasmatic, tuberomammillary, supramammillary and dorsal raphe nuclei (data not shown). lo, lateral olfactory tract; 3v, third ventricle. Scale bars: **a**, **c**, 200 μm; **b**, **d**, 500 μm; **e**, **f**, 50 μm. No Fos immunoreactivity is present in any of the regions observed in the control rats (data not shown).

named ghrelin receptor. Fos also was highly expressed in the dentate gyrus and hippocampus (Fig. 4b; CA1, CA2 and CA3) where ghrelin receptor messenger RNA is abundantly present. The possible involvement of ghrelin in learning and memory requires further investigation. No significant Fos expression was found in the neocortex nor in the cerebellum. Fos distributions were similar in the 0.01, 0.5 and 2 nmol ghrelin-injected rats (data not shown).

The arcuate nucleus is critical for feeding and body weight regulation because it has the leptin-responsive orexigenic neuropeptides, NPY[14,15] and AGRP[16], and the leptin-responsive anorexic neuropeptides, pro-opiomelanocortin[17], and cocaine- and amphetamine-regulated transcript[18]. Of the three rats examined by double immunohistochemistry, ghrelin administration induced Fos expression in 39 ± 6% of NPY neurons in the medial part of the arcuate nuclei (Fig. 4e, f), which is consistent with previous findings that NPY neurons have GHS receptors[19] and express Fos in response to GHS administration[20,21].

We investigated the functional relationship between ghrelin and NPY by blocking either of the peptides in ghrelin- or NPY-induced feeding. Y1 and Y5 receptors are involved in feeding regulation by NPY[22,23]. We first determined the doses of anti-NPY IgG and two antagonists for Y1 and Y5 receptors that are needed to block NPY-induced feeding, while inducing no other unusual behaviour (Fig. 5a). ICV administration of 1 μg anti-NPY IgG 4 h before ghrelin administration cancelled ghrelin-induced feeding; co-administration of two antagonists for Y1 and Y5 receptors also cancelled ghrelin-induced feeding (Fig. 5b). In contrast, anti-ghrelin IgG did not affect NPY-induced feeding (Fig. 5a). Because

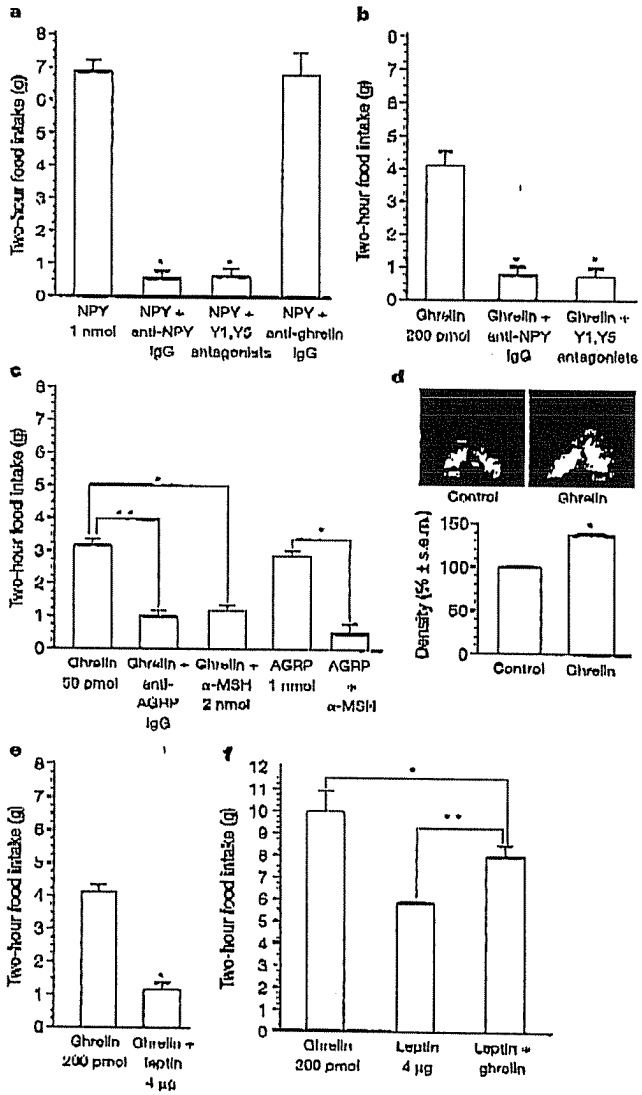

Figure 5 Interactions of ghrelin with NPY, AGRP and leptin. **a**, Effects of administration of anti-NPY IgG, anti-ghrelin IgG or co-administration of Y1 and Y5 antagonists on NPY-induced (1 nmol) feeding. Asterisk, $P < 0.0001$ versus NPY-injected group. **b**, Effects of anti-NPY IgG and co-administration of Y1 and Y5 antagonists on ghrelin-induced (200 pmol) feeding. Asterisk, $P < 0.0001$ versus ghrelin-injected group. **c**, Effects of anti-AGRP IgG or α-MSH on ghrelin-induced feeding. Asterisk, $P < 0.001$; double asterisk, $P < 0.0005$. **d**, *In situ* hybridization of hypothalamic NPY mRNA in rats ($n = 8$ per group) receiving ICV administration of ghrelin (1 nmol) or control vehicle. The quantitative image analysis of NPY mRNA expression in the arcuate nucleus is shown (bottom). Asterisk, $P < 0.01$. **e**, Suppressive effect of leptin on ghrelin-induced (200 pmol) feeding. Asterisk, $P < 0.0001$. **f**, Inhibitory effect of ghrelin on leptin-induced feeding reduction. Rats that had fasted for 8 h received an ICV administration of ghrelin (200 pmol), leptin (4 μg) or leptin followed 1 h later by ghrelin. Asterisk, $P = 0.031$; double asterisk, $P = 0.016$.

AGRP co-localizes with NPY in arcuate nucleus neurons[7,14], we studied the relationship between ghrelin and AGRP in feeding regulation. Ghrelin-induced feeding was suppressed on both treatment with α-melanocyte-stimulating hormone (α-MSH), a melanocortin receptor agonist, and blocking of AGRP, a receptor antagonist[24], with anti-AGRP IgG (Fig. 5c). These results indicate that inhibition of endogenous NPY and AGRP may modulate ghrelin-induced feeding, which suggests that ghrelin interacts anatomically and/or functionally with the pathways of these two peptides.

Inhibition of NPY synthesis and release is a chief mechanism of food-intake reduction mediated by leptin[25,26]. The hypothalamic NPY mRNA level in quantitative *in situ* hybridization[27] increased after ghrelin administration (Fig. 5d). Ghrelin-induced feeding in the light phase was suppressed by an ICV administration of leptin (Fig. 5e). Leptin reduced feeding in fasted rats, whereas ghrelin substantially blocked this reduction in rats that were pretreated with leptin (Fig. 5f). These results indicate that ghrelin may antagonize leptin action in the regulation of the NPY system.

Central ghrelin is a new physiological regulator of nutritional homeostasis. The classic effects of growth hormone in promoting growth of soft tissue, such as bone and cartilage, together with the orexigenic effect of ghrelin suggest that central and peripheral factors activated by ghrelin may underlie growth processes in an integrated manner. Further investigations of ghrelin's function will help our understanding of physiological feeding mechanisms and should facilitate the study of eating disorders.

Methods

Animals

We maintained male Wistar rats under controlled temperature and light conditions (light on 07:00–19:00). We performed cannulation and ICV administration as described[28]. We repeated all of the experiments two or three times. All the compounds were dissolved in 0.9% saline, and 10 μl solution in total was administered. We performed all procedures in accordance with the Japanese Physiological Society's guidelines for animal care.

Feeding experiments

First, various doses of rat ghrelin (Peptide Institute), GHRP-6 (Phoenix Pharmaceuticals) or ghrelin + [D-Lys-3]-GHRP-6 (Peninsula Laboratories) were administered by ICV injections to rats (n = 16–20 per group) weighing 300–325 g at 08:45. Ghrelin (200 pmol) also was administered by ICV injection at 08:45 to rats (n = 12) that had fasted for 8 h. We re-weighed chow 2 h after peptide administration, and calculated food intake. Second, ghrelin (200 pmol) or saline was administered by ICV injection at 18:45 to free-feeding rats (n = 12 per group), after which dark phase (19:00–07:00) food intake was measured. Third, ghrelin (250 pmol per 14 μl saline per day, for 12 d) or vehicle was infused continuously through osmotic minipumps to 7-week-old Wistar rats (n = 10 per group). Cannulae implanted into the lateral ventricles were connected to minipumps (Alzet, type 2002) inserted under the skin of the neck. We measured body weight and food consumption daily at 07:00. On day 12, the rats were killed, and the truncal blood was sampled. Fourth, ghrelin (200 pmol) was administered by ICV injection to 16-week-old spontaneous dwarf rats (SDR) (n = 6) weighing 95–100 g (Japan SLC), after which 2-h food intake was measured.

To investigate the functional relationship between ghrelin and NPY or AGRP, ghrelin was co-administered with an antagonist or antibody for either of the peptides. Rats (n = 12–16 per group) were administered an ICV injection in the morning with the following reagents: ghrelin (200 pmol); ghrelin + anti-NPY IgG (1 μg; Peptide Institute); ghrelin + 1229U91 (30 μg, a Y1 antagonist[29]) + L-152,804 (30 μg, a selective Y5 antagonist[30]); NPY (1 nmol); NPY + 1229U91 + L-152,804; NPY + anti-ghrelin IgG (0.5 μg); NPY + anti-NPY IgG; ghrelin + anti-AGRP IgG (1 μg, Phoenix Pharmaceuticals); ghrelin + α-MSH; AGRP (1 nmol) or AGRP + α-MSH (2 nmol). Doses of each reagent used were the same among the sets except for ghrelin (20, 50 and 200 pmol) + α-MSH (1, 2 and 4 nmol). We injected IgG 4 h before peptide administration in all cases. We monitored food intake for 2 h.

We conducted two experiments to study the interaction between ghrelin and leptin in feeding regulation. First, free-feeding rats (n = 12 per group) were administered an ICV injection in the morning with ghrelin (200 pmol) or ghrelin + mouse leptin (4 μg, a gift from the National Hormone and Pituitary Program). Second, rats (n = 12 per group) that had fasted for 8 h were administered an ICV injection in the morning with ghrelin (200 pmol), leptin (4 μg) or ghrelin + leptin. We injected leptin at 07:45 and other peptides at 08:45 in both of the experiments. We measured 2-h food intake. We analysed groups of data (mean ± s.e.m.) using ANOVA (analysis of variance) and *post hoc* Fisher's test.

Immunoneutralization

We subjected anti-ghrelin antiserum[1] to Affi-gel protein A affinity and then CNBr-Sepharose-coupled ghrelin affinity chromatography. We determined the amount of purified IgG by using a DC protein assay kit (Bio-Rad). First, a 10 μl saline solution of purified anti-ghrelin IgG or preimmune serum IgG from the same rabbit was administered by ICV injection at 08:45 the following morning to rats (n = 10 per group) that had fasted for 8 h. Second, IgG was given through ICV to free-feeding rats (n = 10 per group) at 17:00. We measured food intake after IgG administration.

Locomotor activity

Movement of rats (n = 10 per group) that had been given a continuous ICV infusion of ghrelin (250 pmol per 14 μl saline, for 5 d) or the vehicle through osmotic minipumps was measured on days 1–5 as described[31]. We made locomotor-activity counts every 15 min and summed them for the dark and light phases.

c-*fos* expression

We studied four rat groups (n = 3 per group; 10 pmol ghrelin, 500 pmol ghrelin, 2 nmol ghrelin and 0.9% saline). We administered an ICV injection of ghrelin or saline 90 min before perfusion. Frozen serial brain sections (40 μm thick) were incubated for 2 d with goat anti-c-Fos antiserum (Santa Cruz Biotechnology; final dilution 1:1,500)[32]. We stained the sections by the avidin–biotin complex method[17]. We subjected some sections of the arcuate nucleus to Fos staining, and then to double staining with rabbit anti NPY-antiserum (DiaSorin; final dilution 1:4,000).

In situ hybridization

We performed *in situ* hybridization of NPY mRNA with a 45-nucleotide antisense probe as described[27]. We analysed the images in an MCID imaging analyser[33].

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Identification of the haemoglobin scavenger receptor

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Intravascular haemolysis is a physiological phenomenon as well as a severe pathological complication when accelerated in various autoimmune, infectious (such as malaria) and inherited (such as sickle cell disease) disorders[1]. Haemoglobin released into plasma is captured by the acute phase protein haptoglobin, which is depleted from plasma during elevated haemolysis[1]. Here we report the identification of the acute phase-regulated and signal-inducing macrophage protein, CD163, as a receptor that scavenges haemoglobin by mediating endocytosis of haptoglobin–haemoglobin complexes. CD163 binds only haptoglobin and haemoglobin in complex, which indicates the exposure of a receptor-binding neoepitope. The receptor–ligand interaction is Ca^{2+}-dependent and of high affinity. Complexes of haemoglobin and multimeric haptoglobin (the 2-2 phenotype) exhibit higher functional affinity for CD163 than do complexes of haemoglobin and dimeric haptoglobin (the 1-1 phenotype). Specific CD163-mediated endocytosis of haptoglobin–haemoglobin complexes is measurable in cells transfected with CD163 complementary DNA and in CD163-expressing myelo-monocytic lymphoma cells.

Metabolism of haemoglobin (Hb), the most abundant protein in erythrocytes and blood, is a main function of tissue macrophages, which can engulf senescent erythrocytes (extravascular haemolysis) or take up haemoglobin released from ruptured erythrocytes (intravascular haemolysis) and immature erythrocytes in the bone marrow. Efficient removal of free Hb is essential for health because of the oxidative and toxic properties of the iron-containing haem in Hb. In the macrophage, haem is converted to bilirubin and iron[1].

Whereas various receptors[2–5] for direct or indirect binding of surface-exposed phosphatidyl-serine are suggested to be involved in the recognition and uptake of senescent erythrocytes, the cellular structure involved in the clearance of plasma Hb by macrophages has remained unknown. The plasma protein haptoglobin (Hp) is thought to be involved in promoting the clearance of plasma Hb[9], because it strongly binds free Hb and is depleted during elevated haemolysis[1]. To identify the molecular recognition events determining the clearance of Hb released into plasma during intravascular haemolysis, we constructed an Hp–Hb affinity matrix for the purification of a putative receptor for this complex. Subsequent affinity chromatography of solubilized membranes from three macrophage-containing human tissues (placenta, liver and spleen) yielded a protein with relative molecular mass of 130,000 (M_r 130K) (Fig. 1a). Matrix-assisted laser-desorption ionization (MALDI) mass spectrometry of a tryptic digest of the 130K protein (Fig. 1b) identified it as the scavenger receptor cysteine-rich domain protein, M130/CD163 (refs 10–12), which is an acute phase-regulated transmembrane protein that is expressed exclusively in monocytes (low expression) and tissue macrophages (high expression)[13]. Consistent with the difference in macrophage content of the source tissues for the affinity chromatography, the highest yield was obtained from the spleen (~0.1–0.2 mg CD163 per g membrane). The yields from liver and placenta were about 4 times and 20 times lower, respectively. Isolated CD163 from any of the tissues was of very high purity, and no other proteins, including liver- or placenta-specific proteins, were detected at significant levels. We identified a protein that had an electrophoretic mobility identical to that of CD163 by ^{125}I-labelled Hp–Hb blotting of solubilized spleen membranes (Fig. 1c, lane 4). We confirmed the identity of the Hp–Hb-binding protein as CD163 by immunoblotting (Fig. 1c, lanes 5–8) with two different monoclonal antibodies against CD163 (refs 13 and 14).

Haptoglobin is synthesized as a single chain, which is cleaved to an amino-terminal α-chain and a carboxy-terminal β-chain. The basic structure of Hp, as found in most mammals, is a homodimer (Fig. 2a) designated Hp(1-1) in which the two Hp molecules are linked by a single disulphide bond through their respective ~9K α-chains[15]. In humans, a variant with a longer α-chain is also present in all populations. This variant arose apparently by an early intragenic duplication, presumably originating from an unequal crossover of two basic alleles, resulting in an Hp with an α-chain of ~14K. The short and long α-chains are designated as α^1 and α^2, respectively. As the cysteine forming the intermolecular disulphide bond between the α-chains is also duplicated, humans homozygous for the long variant allele show a multimeric Hp phenotype (Fig. 2a) designated Hp(2-2). Hp(2-1) refers to the phenotype (both Hp dimers and multimers) seen in humans heterozygous for the two variant alleles.

Analysis of Hp–Hb complexes binding to immobilized CD163 showed a high-affinity binding of both dimeric and multimeric Hp–Hb complexes (Fig. 2b, c). Figure 2b shows a surface-plasmon resonance analysis of CD163 binding of the dimeric Hp(1-1)–Hb complex and the multimeric Hp(2-2)–Hb complex. No binding of non-complexed Hb (Fig. 2b, left panel), Hp(1-1) (Fig. 2b, middle panel) nor Hp(2-2) (Fig. 2b, right panel) was detected, thus indicating that a neoepitope for receptor binding is exposed in the Hp–Hb complex. Accordingly, maximal receptor binding was measured when the Hb-binding capacity of Hp reached saturation at equimolar concentrations of Hb and Hp (Fig. 2b, middle and right panels). The Hp(2-2)–Hb complex yielded a higher response and the dissociation was slower as compared with the Hp(1-1)–Hb complex. The results shown in Fig. 2b were obtained using the A_0 ($\alpha 2\beta 2$) form of Hb. We obtained similar results using the A_2 ($\alpha 2\delta 2$) form, or the S form (Hb with the mutation for sickle-cell disease)[16] (data not shown).

We used a solid-phase assay with immobilized CD163 in micro-